# Simplified and Efficient Labeling of Human Platelets in Plasma Using Indium-111-2-Mercaptopyridine-*N*-Oxide: Preparation and Evaluation

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The need for a gamma-emitting radioactive agent that will label platelets in plasma efficiently by a procedure that can be used uniformly in each laboratory is well recognized. A water soluble sodium salt of 2-Mercaptopyridine-*N*-oxide (Merc) was evaluated that quantitatively chelated $^{111}$In at a pH range of 0.7 to 7.4, and allowed greater than 80% incorporation of $^{111}$In in platelets in plasma. This was dependent on pH, Merc concentration, and platelet concentration. Platelets were labeled using either preformed [$^{111}$In]Merc or incubating platelets with 2.5 $\mu$g dry Merc first and then with $^{111}$In. The latter method provided a simple kit procedure that labeled platelets with negligible alteration of in vitro aggregability. In dogs, labeled platelets had normal survival (7.5 days), 66 ± 6.6% recovery, detected vascular thrombi (thrombi/blood = 59.4), and pulmonary emboli (PE/blood = 46.2) by scintigraphy. In the kit procedure, the use of Merc compared favorably to that of oxine and tropolone.

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Ever since the first report in 1976, indium-111 ($^{111}$In) oxine-labeled platelets have been used extensively in a variety of experimental and clinical applications (*1–14*). Although results have been highly encouraging, there is a growing consensus among users that the in vitro aggregability and in vivo viability of platelets are better preserved when the labeling procedure is performed in autologous plasma (*4,15–18*). Due to the chelation of indium with plasma transferrin (*19*) and other lipoproteins (*20*), the labeling of platelets with [$^{111}$In]oxine in plasma is inefficient. An efficient labeling of human platelets can be achieved in normal saline, but only at the expense of their viability (*4,15*). This has compelled each investigator to modify the original nonplasma labeling technique to allow efficient incorporation of radioactivity and yet to minimize adverse effects on platelet viability, as well as to make the procedure convenient for handling in the laboratory (*13,30–23*). Consequently, each procedure has become an individualized method using a variety of salt-balanced media, different proportions of various anticoagulants, and various centrifugal forces, all of which are known to affect platelet viability (*15,24*). This has caused a large variation in the quantities of radioactive platelets distributed in vivo, and has made comparisons of such very basic information from different laboratories impossible (*25*). The development of new $^{111}$In agents such as acetylacetone or tropolone has not solved the problem since the use of a nonplasma medium is still reported to be desirable for an efficient cell labeling (*17,18,26*).

The aim of this investigation was to develop a new agent that would allow efficient labeling of human platelets in plasma and provide a simple procedure that could be conveniently and uniformly followed in every laboratory.

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The efficacy of the new agent was compared to that of oxine and tropolone, and platelets labeled with $^{111}$In by the new procedure were evaluated in vitro and in experimental animals.

## MATERIALS AND METHODS

### Preparation of radioactive agents and separation of platelets

*The new agent: Properties, preparations, and stability*

The agent 2-Mercaptopyridine-*N*-oxide (Merc), also known as pyrithione or omadine, contains, like oxine, two functional groups (*N*-oxide and SH), which make the compound an excellent metal chelating agent (*27*). Sodium salt of Merc* is more readily soluble in aqueous solvents than its acid form† and is less toxic (mice, i.v. $LD_{50}$, 335 ± 15 mg/kg and rats, i.p. $LD_{50}$ 745 mg/kg) than oxine (mice, i.p., $LD_{50}$, 88.8 mg/kg) (*28*, *29*).

*Preparation of [$^{111}$In]Merc*

Indium-111 Merc was prepared by adding 50 μg of the chelating agent (1 mg/ml aqueous solution) to a series of acid-washed, glass test tubes containing $^{111}$In in 1 ml acetate or citrate buffer solutions (pH 0.7 to 7.4) made in deionized water. The radioactivity was then extracted twice in 1 ml chloroform. On several occasions, radioactivity in 1 ml aliquot of [$^{111}$In]Merc prepared either in 0.15*M* NaCl, 3.8% Na citrate, 0.05*M* phosphate buffer (pH 7.4), or 0.1*M* acetate buffer (pH 7.4) and stored at room temperature for up to 15 days was extracted in chloroform. The radioactivity in aqueous and nonaqueous phase was then measured.‡

*Preparation of dry Merc*

In another set of experiments, 2 μg of Merc acid or 2.5 μg Merc Na salt were dispensed in 5 ml polystyrene (not polypropylene) test tubes,§ air dried and stored for subsequent use. ~750 μCi of [$^{111}$In]chloride¶ and 1/3 volume of 1*M* citrate buffer pH 6.5 were dispensed in another tube as required and either dried using a gentle stream of argon, or used as such.

*Separation of platelets*

Studies, with the exception of those involving canine models, were carried out using human platelets separated from venous blood of normal healthy volunteers who had not taken any medication for at least 1 wk prior to donating blood. Two blood samples, 34 ml in 6 ml anticoagulant A (5 g dihydrated trisodium citrate and 2.98 g monohydrated citric acid dissolved in 200 ml distilled water and sterilized) and 15 ml in 1.5 ml anticoagulant B (3.8% trisodium citrate) were drawn. The composition and storage conditions of the anticoagulants and the centrifugation procedure for harvesting platelets were identical to those described previously (*15*). Briefly, blood A was divided in two equal volumes in 50-ml sterile, conical, polypropylene tubes and B was transferred to a third. Platelet-rich plasma (PRP) was obtained by centrifugation at 180 *g* for 15 min in a calibrated, horizontal swing rotor, table-top centrifuge. A 0.5-ml aliquot of PRP B was removed and stored at 22°C as a control for subsequent aggregation studies. PRP A-combined and PRP B were then centrifuged at 1,000 *g* for 10 min. Platelet-poor plasma (PPP) B was removed and stored at 37°C. All but 1.5 ml (Method I) or 0.5 ml (Method II) PPP A were also removed and platelets in button A were resuspended for labeling. Platelet concentration was measured using a Coulter counter. Aggregation studies were carried out using a single channel Chronolog aggregometer. A 480 μl aliquot of PRP B was used as a control. A similar aliquot of labeled platelets resuspended to the initial concentration in PPP B was used as a study sample. Both samples were incubated at 37°C, stirred with a Teflon-coated magnetic bar (1,000 rpm) and stimulated with 20 μl of 1 m*M* adenosine diphosphate.

*Indium-111 oxine, tropolone, and acetylacetone*

Indium-111 oxine was obtained commercially,** and [$^{111}$In]tropolone and acetylacetone were prepared as described previously (*18*,*26*). For comparison in Method II, separate sets of test tubes were prepared and a known quantity (2–50 μg) of oxine dissolved in ethanol and that of tropolone dissolved in deionized water were dispensed. Solvents were then evaporated by blowing a gentle stream of argon. Twenty to 50 μCi $^{111}$In were also prepared as described previously.

### Labeling platelets in plasma (Method I)

In this method, platelets in button A suspended in 1.5 ml PPP A were incubated with preprepared [$^{111}$In]Merc, [$^{111}$In]oxine, or [$^{111}$In]tropolone. At the end of a predetermined incubation period, platelets were centrifuged at 1,000 *g* for 10 min, supernatant removed, radioactivity measured, and percentage associated with platelets was calculated. Labeled platelets were then washed at least once in PPP A, to ascertain that the radioactivity was firmly associated with platelets. Labeled platelets were then resuspended in PPP B for aggregation studies.

*Influence of Merc concentration on labeling efficiency and platelet aggregability*

Five hundred microliters [$^{111}$In]Merc in 0.05*M* phosphate buffer pH 6.5 were added to a series of test tubes containing ~$1.6 \times 10^9$ platelets in 1.5 ml PPP A in such a way that the Merc concentration in platelets varied between 1 to 25 μg/ml. Platelets were then incubated at 22°C for 20 min, centrifuged, washed once, and resuspended in PPP B for the determination of labeling yields and platelet aggregability.

*Influence of incubation time and temperature*

Three test tubes each containing ~9 × $10^9$ platelets suspended in 2 ml PPP A were incubated either at 4, 22, or 37°C. Each test tube then received [$^{111}$In]Merc so that a final Merc concentration was 5 μg/ml. At predetermined times varying between 5 to 70 min, 100 μl aliquot from each test tube were transferred to separate test tubes, each containing 1 ml of PPP A. Platelets were then centrifuged immediately and washed, and labeling efficiency was determined.

*Influence of pH on labeling yields*

In order to be able to hold pH of the media constant during incubation period, 0.9% NaCl was used as a suspending medium and pH was adjusted between 4.46 to 6.97 by adding either 0.1*M* citric acid or 0.1*M* sodium citrate. Each milliliter of suspending medium contained 8 × $10^8$ platelets and ~50 μCi $^{111}$In chelated with 5 μg Merc. Incubation was carried out at 22° for 20 min after which platelets were centrifuged and associated radioactivity was measured.

*Platelet concentration and labeling efficiency*

Human platelets varying between 4 × $10^8$ and 1.2 × $10^{10}$ were suspended in 1.5 ml PPP A, and incubated at 22°C for 20 min with 500 μl aqueous solution containing ~50 μCi $^{111}$In chelated with 10 μg Merc. One and one half milliliters PPP A, without added platelets, and incubated with [$^{111}$In]Merc served as a control. Following centrifugation and a PPP A washing, labeling efficiency was determined in the usual manner.

**Labeling platelets in plasma (Method II)**

In Method II, platelets in button A were suspended in 0.5 ml, instead of 1.5 ml PPP A used in Method I. Generally, they were then transferred to a polystyrene test tube containing dry Merc, prepared and stored as described above and incubated at 22°C for 10 min. These platelets were then transferred to another test tube which contained $^{111}$In in 0.25*M* citrate buffer pH 6.5, either dry or in solution. Following further incubation for a predetermined period of time, platelets were centrifuged and washed, and labeling efficiency determined. Platelets were then suspended in PPP A for injection or diluted fivefold in PPP B for aggregation studies.

The following studies carried out in Method I were repeated in order to establish the optimal labeling conditions in Method II.

*Influence of quantity of dry Merc*

To a series of test tubes containing 2 to 50 μg dry Merc were added 0.5 ml platelet suspension in PPP A. Platelets were incubated for 5 min and transferred to each of several test tubes containing $^{111}$In in 0.25*M* citrate buffer. They were further incubated at room temperature for 20 min, centrifuged, washed, and assayed for radioactivity to determine labeling efficiency.

*Influence of incubation time*

Each of seven test tubes containing 2.0 μg Merc received 0.5 ml platelet suspension in PPP A. Platelets were incubated at room temperature for 5 min and transferred to other sets of test tubes containing dry $^{111}$In in 0.25*M* citrate buffer. They were then incubated for predetermined periods of time at 22°C or at 37°C and centrifuged. Labeling efficiency was then determined.

*Comparison with Merc, oxine, and tropolone*

Three sets of test tubes containing 2 to 50 μg Merc, oxine, or tropolone each received 0.5 ml of platelet suspension in PPP A. They were then incubated with $^{111}$In as before and the quantity of radioactivity incorporated was determined.

**In vivo evaluation in dogs**

*Platelet recovery and survival*

Autologous platelets labeled with 300–350 μCi [$^{111}$In]Merc (Method I) were given to one group of three dogs and those labeled by Method II to another group of three dogs. Two sets of blood samples were drawn from each animal at 5 min, 30 min, 1 hr, 2 hr, and 4 hr after injection on Day 1 and then once daily for 7 days. In set one, ~ 2-ml blood samples were drawn without anticoagulant, and transferred to weighed test tubes. These were stored for radioactivity counting on Day 7 along with two $^{111}$In standards prepared on the day of injection. These test tubes were weighed again and radioactivity in each test tube was counted using Beckman automatic gamma counter. The count rate was then normalized for a unit weight of blood and expressed as % administered dose. The total blood volume in each animal was estimated as 7.6% of the animals' body weight (*30*). Recovery, i.e., the percentage radioactivity remaining in circulation, was calculated by multiplying the total volume of blood, in milliliters, by % radioactivity in a gram of blood drawn at 5 min postinjection.

Assuming the radioactivity in the 5-min blood sample as 100%, percentage of radioactivity in other blood samples was calculated and plotted on a linear scale against time.

In set number 2, ~ 5 ml blood drawn in 0.8 ml acid citrate dextrose (ACD) as an anticoagulant was subjected to centrifugation to separate platelets, other cells, and plasma. Radioactivity in each fraction was then counted and relative percentage of radioactivity in the three fractions of each sample was determined.

*Imaging experimental venous thrombi and pulmonary emboli*

Venous thrombi were induced in a deep femoral vein of three dogs, by altering the intima from passage of 1.5

mA direct current for 1 hr (*1,31*). Two hours following the vessel wall damage, ~400 μCi of [$^{111}$In]Merc platelets labeled using Method I were given to the animals. Periodical gamma camera images were obtained between 45 min to 3 hr postinjection. Damaged veins were then dissected, thrombi separated, weighed, and radioactivity associated with thrombi, vessel, and blood drawn at the time of dissection were counted. The observed count rate in each sample was then normalized for unit weight and thrombus/vessel and thrombus/blood ratios were calculated.

In one additional animal, pulmonary emboli were induced using the technique described by Sostsman et al. (*32*). Forty-eight hours following the procedure, ~450 μCi $^{111}$In-labeled autologous platelets were administered. Animals were then imaged for 3 hr, killed with an overdose of pentobarbital, and pulmonary emboli (PE), lung, and blood samples were obtained for the measurement of concomitant radioactivity.

## RESULTS

### Preparation of radioactive agents and separation of platelets

In the presence of Merc, greater than 90% of added $^{111}$In radioactivity was extractable in chloroform at pH between 0.7 and 7.4 (*33*) (at pH 7, only 13 ±4% of added sulfur-35 ($^{35}$S) Merc was extractable in chloroform).

The percentage of extractable [$^{111}$In]Merc, stored at room temperature for 2 wk remained unchanged when Merc concentration was greater than 5 μg/ml. As the concentration of Merc decreased, the extractability of [$^{111}$In]Merc decreased.

The presence of up to 3.3 molar excess of bivalent cations such as $Zn^{2+}$, $Cd^{2+}$, and $Fe^{2+}$ did not alter the percentage of extractable [$^{111}$In]Merc. However, in presence of as little as 1/3 molar $Fe^{3+}$ ($In^{3+}$:$Fe^{3+}$::1:0.3) the extraction efficiency dropped to 70%.

~$8.9 \pm 6.2 \times 10^9$ Platelets were harvested from 34 ml of venous blood from healthy human volunteers. Leukocyte and erythrocyte contamination in these platelets averaged less than one for every $10^5$ and $10^4$ platelets, respectively.

**TABLE 1**
Effect of pH on Platelet Labeling Efficiency

| pH | % Yield |
|---|---|
| 4.46 | 28.9 ± 12.5 |
| 6.36 | 81.2 ± 6.2 |
| 6.46 | 90.7 ± 3.4 |
| 6.97 | 98.4 ± 1.3 |

* Platelets ($8 \times 10^8$) were suspended in 0.9% NaCl and pH was adjusted with citric acid or sodium citrate.

### Labeling platelets in plasma (Method I)

Experiments with increasing concentrations of Merc revealed that for optimal labeling efficiency, Merc concentration of 5 μg/ml and 20 min incubation were required. As the Merc concentration increased to >5 μg/ml, the labeling efficiency decreased, but had no adverse effect on aggregability of labeled platelets. The yield at 4°C was consistently lower than that obtained at 22°C or 37°C. The yield at 37°C was higher than those at 22°C at all incubation times, but not high enough to warrant choosing 37°C as an incubation temperature (Fig. 1).

The labeling yield was maximum at neutral pH and decreased with decreasing pH (Table 1). The labeling efficiency also increased as platelet concentration increased and was consistently higher than that with [$^{111}$In]oxine used under identical conditions (Fig. 2). However, it was unaffected in the presence of citrate in concentrations of up to 35 m*M*.

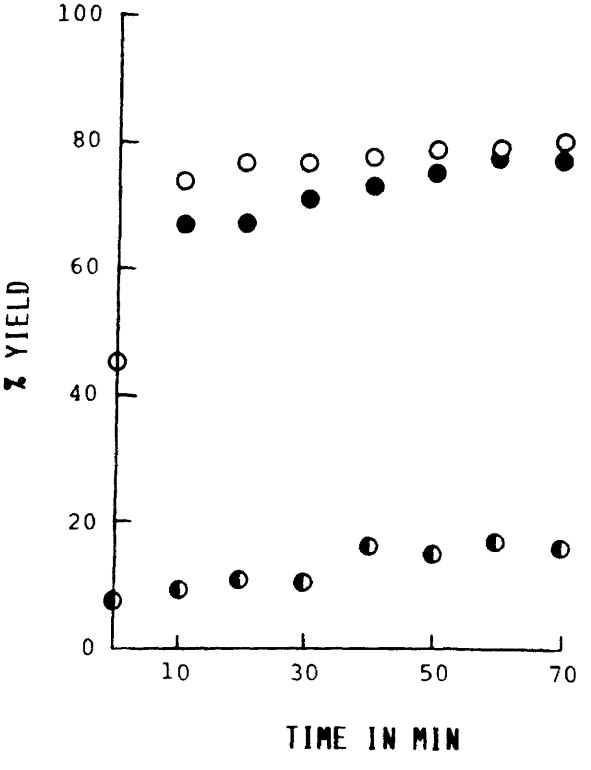
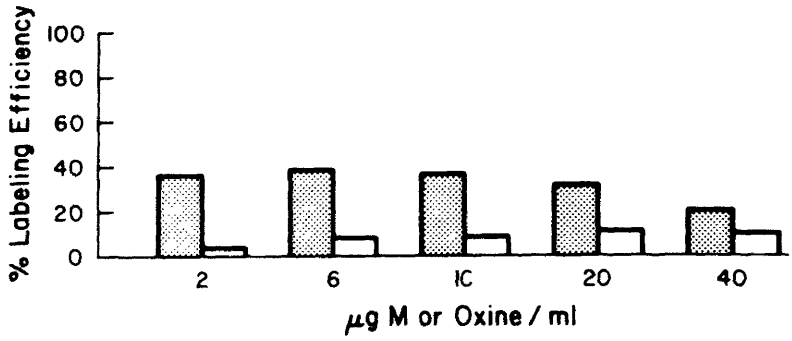



**FIGURE 1**
Influence of temperature on labeling yield. ~$9 \times 10^9$ platelets/ml PPP A were labeled by Method I. (◐) 4°C; (●) 22°C; (○) 37°C. Incubation at 37° enhanced labeling yield but not high enough to warrant incubation at this temperature

**FIGURE 2**
~5.6 × $10^8$ Platelets were suspended 1 ml PPP A and incubated for 20 min at 22°C either with [$^{111}$In]Merc ([$^{111}$In]-M), or with [$^{111}$In]oxine at concentration shown (Method I). Platelet concentration = 5.6 × $10^8$/ml. (▩) [$^{111}$In]M: (□ [$^{111}$In]oxine. Labeling efficiency using [$^{111}$In]Merc was consistently higher. (At higher platelet concentrations, higher labeling efficiencies are achieved with [$^{111}$In]-Merc. M represents Merc)




### Method II

Consistent with Method I, the optimal Merc concentration in this dry Merc method was also 5 μg/ml platelet suspension. This is evident in Fig. 3, which indicates that the maximum incorporation of radioactivity occurred when 2.0 μg Merc was incubated with platelets suspended in 0.5 ml PPP A. We observed that incubating platelets at 37°C in this method, too, offered no particular advantage and that 15-20 min incubation was long enough. The use of 2-50 μg dry oxine or tropolone labeled platelets poorly and clearly indicated the advantage of using Merc (Fig. 4).

### In vivo evaluation

The survival of platelets labeled by both methods was studied in dogs. The recovery of platelets at 15 min postinjection averaged 65 ± 3.5% and 66 ± 6.6% by Methods I and II, respectively. Although the percentage of labeled platelets in circulation at a given point generally varied in each animal, the survival of platelets by both methods was ~ 7.5 days. Figure 5(upper) indicates the ability of [$^{111}$In]Merc-labeled autologous platelets (Method I) to scintigraphically detect 2-hr-old experimental thrombi, at 40 min postinjection. In three animals, thrombi/blood ratios at 3 hr postinjection averaged 59.4.

The 48-hr-old experimental PE was also detectable [Fig. 5(lower)] by in vivo imaging at 90 min postinjection

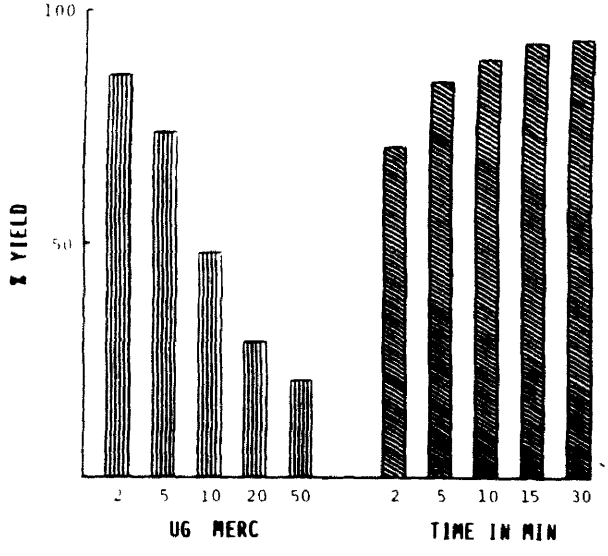



**FIGURE 3**
Composite of results from two separate sets of experiments demonstrate that in Method II maximum labeling yield is obtained using 2 μg dry Merc and 15 to 30 min incubation. ~9 × $10^9$ Platelets suspended in 0.5 ml plasma were incubated with quantities of Merc shown before labeling with $^{111}$In

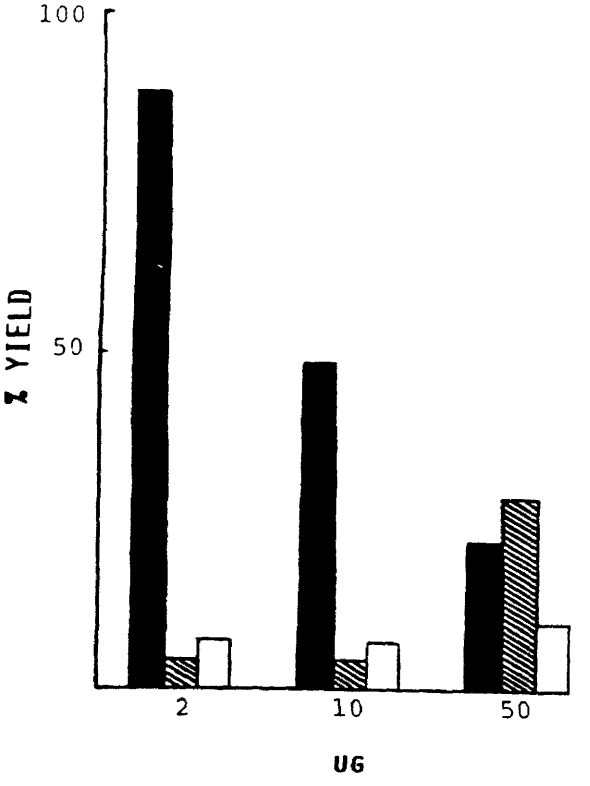



**FIGURE 4**
~9 × $10^9$ Platelets suspended in 0.5 ml PPP A were incubated either with (■) Merc; (▨) oxine; or (□) tropolone (dry), and labeled with $^{111}$In by following Method II. Results indicate that Merc was agent of choice

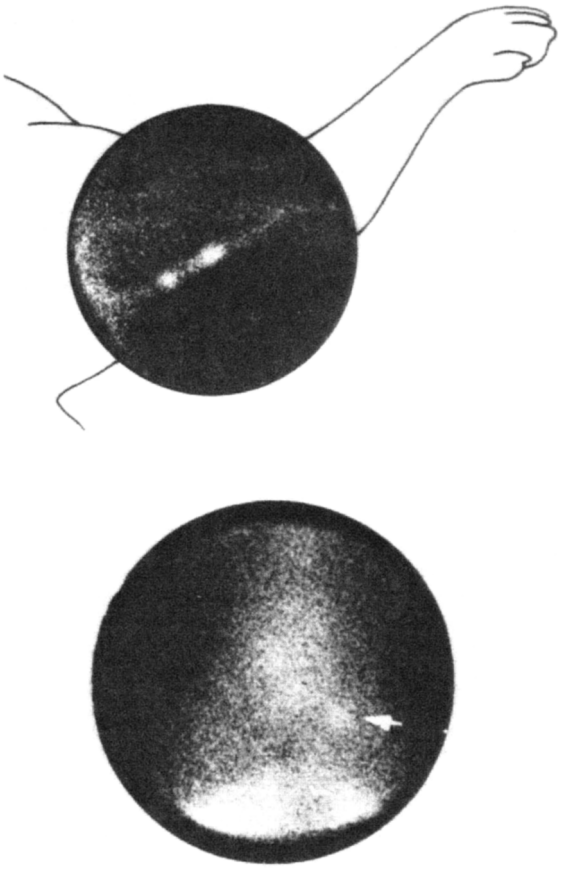


**FIGURE 5**
Upper: Thrombi were induced 2 hr prior to injecting platelets labeled by Method I and imaged at 40 min postinjection. Despite high blood-pool activity, thrombi were delineated and had 59.4 times more radioactivity than in equal weight of blood. (These ratios were higher than those obtained at 24 hours postinjection using platelets labeled with [$^{111}$In]oxine in saline (Ref. *1*). Lower: Anterior gamma camera image of dog which had received [$^{111}$In]Merc platelets 90 min previously. Despite high blood-pool activity at this time, clot (arrow) in left lung base is visible. Also visible are blood-pool activity in heart (above the clot), liver at lower right and spleen at lower left of image

and the PE/lung and PE/blood ratios were 38.7 and 46.2, respectively.

## DISCUSSION

The development of a technique to label circulating platelets with [$^{111}$In]oxine in vitro has prompted many investigators to undertake a variety of experimental and clinical studies that were previously either too complex or impossible to perform. During the course of these studies, investigators have become increasingly aware of the difficulties caused by the inability of [$^{111}$In]oxine to label platelets in plasma. The aim of this work was to develop an agent that would label platelets efficiently in plasma and to provide a method that could be used uniformly in any laboratory. This study demonstrates that the use of [$^{111}$In]Merc satisfies both criteria.

Many parameters such as pH, centrifugal forces, and a variety of chemicals induce structural and biochemical changes in platelets (*34*,*35*). Taking into account previous knowledge and experience, new parameters that might affect platelet viability and labeling yield were studied as a guideline. The Merc and platelet concentration, suspending medium, reduced handling, and time and temperature are all favorable factors in [$^{111}$In]Merc labeling, with perhaps the exception of pH. Although the maximum labeling yield occurred at pH 7, a pH of 6–6.5 (PPP A) was chosen to prevent platelets from forming aggregates. From this point of view, we found anticoagulant A was more satisfactory than the commercially available ACD, since the use of the latter allowed platelet aggregates to form on ~5% of the occasions. Such difficulties were eliminated with the use of ACD-A.

Platelets can be labeled with similar efficacy by two methods. In Method I, they could be suspended in 1.5 ml PPP A and incubated with a 500 $\mu$l aqueous solution of $^{111}$In chelated to 10 $\mu$g Merc. Although this procedure is suitable for use, we prefer Method II which uses 2.0 $\mu$g of dry Merc since it is convenient. Merc dried in sterile polystyrene tubes and stored aseptically in otherwise normal laboratory conditions can be readily used for a period of at least 3 wk. This, of course, does not eliminate the use of 2 $\mu$g Merc dispensed from a freshly prepared (1 mg/ml) aqueous solution.

Indium-111 (chloride in 0.05*M* HCl) can be used in a dry form or in solution diluted with 1/3 of its volume of 1*M* citrate buffer pH 6.5. These procedures eliminate the acidity before incubation platelets with $^{111}$In. In order to keep the volume of $^{111}$In small, which makes it quicker to evaporate or minimizes plasma dilution, we used concentrated $^{111}$In solution commercially available.** The other advantage of this method is the ease by which all ingredients required for labeling could be made available commercially as a kit. The availability of such a kit may eliminate the use of a laminar flow hood. Furthermore, the uniform method would enable investigators to compare their findings, particularly those of platelet kinetics in vivo, in a more realistic manner than currently possible. Results in Fig. 4 indicate that the use of neither oxine nor tropolone offer such a possibility.

Merc is less toxic than oxine and water soluble like acetylacetone and tropolone (*17*,*18*,*26*,*33*). Merc concentration in excess of 5 $\mu$g/ml platelet suspension reduced labeling efficiency, but did not affect platelet aggregability. Platelets labeled with [$^{111}$In]Merc had normal survival, recovery in dogs, and the ability to scintigraphically detect vascular thrombi (Figs. 5 upper, lower). A scintigraphic detection of 2-hr-old venous

thrombi with thrombus to blood ratio of 59.4 at 40 min following platelet administration and the detection of 48-hr-old PE, with a PE/lung and PE/blood ratios 38.7 and 46.2, respectively, at 90 min following administration of labeled platelets were particularly noteworthy since at this time the proportion of circulating radioactivity was high.

The mechanism by which the dry Merc method facilitates platelet labeling is being evaluated using $^{35}S$-labeled Merc. However, our gel filtration studies have clearly indicated that $^{111}In$ binds to at least three cytoplasmic components, of which the major one has an apparent molecular weight of ~5,200 daltons (*36*). This is in agreement with [$^{111}In$]oxine platelet subcellular localizaton studies performed by Baker et al. (*37*) and Hudson et al. (*38*) but contradicts preliminary results of Mathias and Welch which indicate that the $^{111}In$ in platelets was loosely bound to only one component with a molecular weight of ~400,000 daltons (*39*).

The dry method can also be used to label leukocytes. A basic difference, however, is that for optimal labeling efficiency 20 μg Merc is required to incubate with leukocytes suspended in 0.5 ml plasma. The details of these studies are described elsewhere (*40*).

## FOOTNOTES

* Sigma Chemical Company, St. Louis, MO.

† Aldrich.

‡ Capintec ionization chamber, Capintec, Inc., Ramsey, NJ

§ Falcon.

¶ Medi-Physics research grade, 50 mCi/ml, 0.05*M* HCl, Medi-Physics, Richmond, CA.

** Medi-Physics, Richmond, CA.

†† Beckman automatic gamma counter.

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